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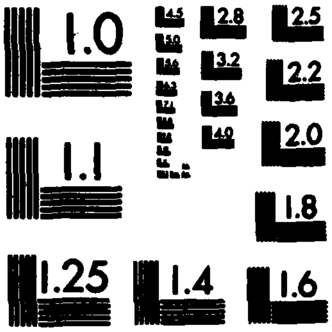

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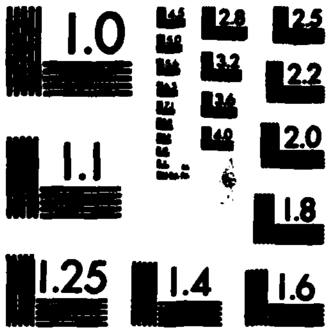

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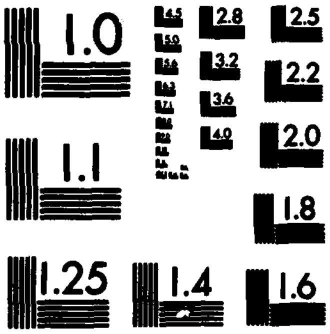

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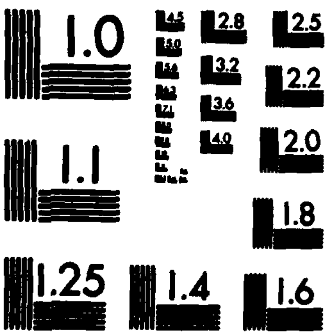

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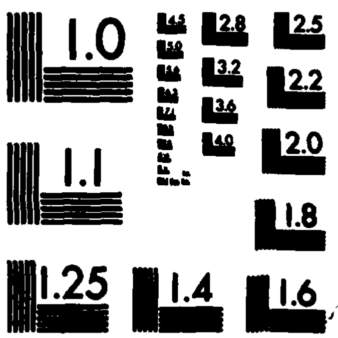

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This contract supports basic research to test the hypothesis that the mechanism of the movement of water and salts across the intestinal epithelium depends not only on the properties of cellular membranes and membrane bound enzymes, but also includes a role for the structural proteins of the cytoplasm. The research plan tests the effect of drugs, which alter the conformational state of the cytoskeletal actin filaments and microtubules, on the transport of water and salts in the rat small intestine.

20. ABSTRACT (continued) micro grams

In Year 1, an in vivo open perfused intestine model was used to screen drugs f their effect on transport. The results showed that cytochalasin B, a drug whi causes the depolymerization of actin filaments, can inhibit the transport of water and sodium across the living intestine at doses from 10-100 µg/ml. Another drug, colchicine, which depolymerizes microtubules inside cells, also inhibited transport at 0.5-2.5 mM doses. Both drugs at higher doses could induce a secretory phenomenon that was not explainable on the basis of tissue breakdown. In addition, the tissue culture medium L-15 was found to support in vivo and in vitro intestinal cell function. In Year 1, four journal papers two chapters in books, and three abstracts credited support from the contract.

During Year 2, major effort has been concentrated on testing this hypothesis o the isolated rat intestinal epithelium in vitro, in the Ussing chamber. Adjacent segments of rat small intestine were mounted in parallel chambers, perfus with nutrient L-15 medium, and bubbled with 95% oxygen at 37° C. In the Ussin chamber it is possible to measure electrophysiological responses of the tissue and isotopic ion fluxes. Tissue morphology was monitored by electron microscopy.

A large number of experiments were conducted to test the effect of cytochalasi B (20 µg/ml in L-15) on sodium transport in the in vitro system. During one hour perfusion with L-15, and one hour perfusion with 0.5% DMSO in L-15, electrical parameters of membrane potential (PD), short circuit current (ISC), and tissue resistance (R) remained constant. Within 20 min of the addition of cytochalasin B (CB), the actin filament depolymerizing agent, both PD and ISC fell dramatically while tissue resistance (R) and tissue morphology remained constant.

^{22}Na isotope flux experiments on parallel tissues indicated net active sodium transport in control states. Mucosal to serosal sodium flux was greatly decreased upon the addition of CB. Passive fluxes were not greatly affected. These results are consistent with a role for actin filaments in water and ion transport, and are submitted for publication in the American Journal of Physiology.

The drug taxol, which drives microtubules into the polymerized state, is being tested currently in the in vivo model, by a minority summer student, and in vitro in the Ussing chamber. Preliminary results suggest a stimulation of transport by taxol in vivo and no effect in vitro.

In the period February 1, 1982 to October 1, 1982, six journal papers, one chap ter, one book, and nine abstracts have been generated which credit support of this contract.

Milestones:

1. Proof that drugs which depolymerize actin filaments can decrease transport in vivo and in vitro.
2. Proof that drugs which affect microtubule organization can affect transport in vivo and in vitro.
3. First demonstration of statistically significant net transport of sodium in vitro in rat small intestine.
4. First demonstration of extended viability of isolated rat small intestinal enterocytes in vitro.

OFFICE OF NAVAL RESEARCH

Contract N00014-81-K-0167

Task No. NR 207-275

TECHNICAL REPORT No. 2

A Role for Cytoplasmic Structural Proteins in the Transport of Water and Salts in the Intestine

by

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September 28, 1982

2nd Annual Progress Report, Contract N00014-81-K-0167

Contents

2nd Annual Progress Report on ONR Contract N00014-81-K-0167

A Role for Cytoplasmic Structural Proteins in the Transport of Water and Salts in the Intestine

February 1, 1982 - October 1, 1982
(Year 2 - eight months)

This report covers the research and publications conducted under Contract N00014-81-K-0167 during the second year of a three year incrementally funded effort. The purpose of this effort is to test the hypothesis that the cytoplasmic structural proteins, tubulin and actin, may play a role in the transport of water and salt across the intestinal epithelium. In Year 1, the research concentrated on the investigation of this hypothesis in the live animal, in vivo model, of the functioning intestine. Previous reports have summarized the findings of this research in which drugs affecting cytoplasmic organization in intestinal enterocytes were shown to inhibit transport (see Technical Report 1, N00014-81-K-0167). The second year of this project has concentrated mainly on the use of an in vitro model, utilizing the intestinal epithelial membrane in the Ussing chamber to continue the testing of the hypothesis.

Year 2 - Results

I. Results of Tests in the In Vitro Model with Cytochalasin B Disruption of Actin Filaments

Work on isolated rat small intestine membrane in the Ussing chamber has been carried out since February 1982, in collaboration with Dr. Linda Shanbour, Associate Professor, and Dr. Karl Karnaky, Assistant Professor, Department of Physiology, University of Texas Medical School, Houston, Texas. Dr. Shanbour has provided expertise gained from years of experience with the gastric mucosa in Ussing chambers to train this investigator in their use for rat small intestine epithelia. She has provided Ussing chambers and electronic monitoring equipment, but all experiments have been conducted by Dr. Beall. Dr. Karnaky is in charge of the Department of Physiology's electron microscopy lab and has provided technical assistance in the examination of intestinal sections. Dr. Beall has examined the normal and drug treated responses of ~30 pieces of intestinal epithelia over the last five months and the results of the project are ready for publication.

a. Experimental Method

Adjacent pieces of rat small intestine from the jejunum are mounted in parallel Ussing chambers, perfused with nutrient L-15 tissue culture medium, and bubbled with 95% O_2-5% CO_2 at 37° C. Electrophysiological parameters of membrane potential (PD), short circuit current (ISC), and tissue resistance (R) are measured with agar bridges and electrodes. Tissue morphology is monitored by electron microscope.

b. Results of Electrophysiological Measurements -- Effects of the Actin Disrupting Agent Cytochalasin B

The results of electrophysiological measurements of the membrane potential (PD), the short circuit current (ISC), and the resistance (R) of rat small intestine are shown in Graph A.

(1) Tissues are able to sustain good PDs without loss of R during the 3 hrs 40 min of the experiments. (See controls.)

(2) During 1 hr of perfusion with L-15 without drugs or DMSO, the PD, ISC, and R remain basically unchanged.

(3) During the second hour when DMSO alone (at < 0.5%) is present, the only change is a slight increase in ISC.

(4) However, within 20 min of the introduction of cytochalasin B at 20 μg/ml L-15, PD and ISC fall to ~50% of their original value, without any significant change in R.

Conclusion

These results alone suggest a reduction in the active movement of ions across the intestinal epithelium by the action of cytochalasin B, without any gross tissue damage.

The type of ion movement inhibited is investigated in Section c and the structure of the tissue in Section d.

c. Results of ^{22}Na Isotopic Flux Measurements

For these experiments, two pieces of adjacent intestine were placed in parallel chambers. High levels (10 μC) of ^{22}Na as NaCl were added to either the mucosal or serosal side of each chamber so that M→S or S→M fluxes of ^{22}NA could be measured and the net sodium flux calculated. The results are shown in Graph B.

(1) During the control period, the net or active sodium transport was around 5 $\mu Eq/cm^2$ of tissue/hr.

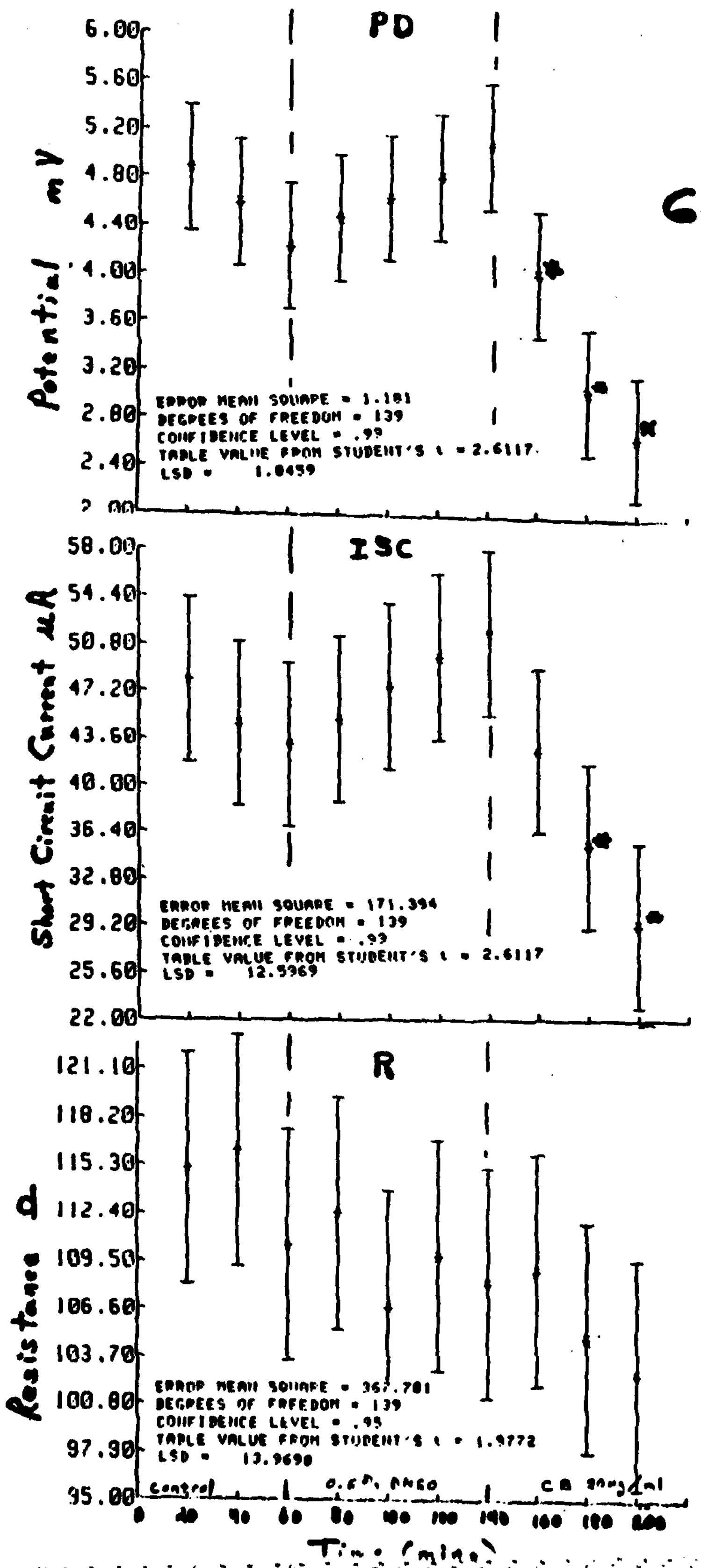
(2) DMSO at < 0.5% did not alter this transport rate significantly.

(3) After the addition of cytochalasin B at 20 μg/ml L-15, there was a rapid decrease in the M→S flux of sodium, without a significant decrease in the passive S→M movements.

Conclusion

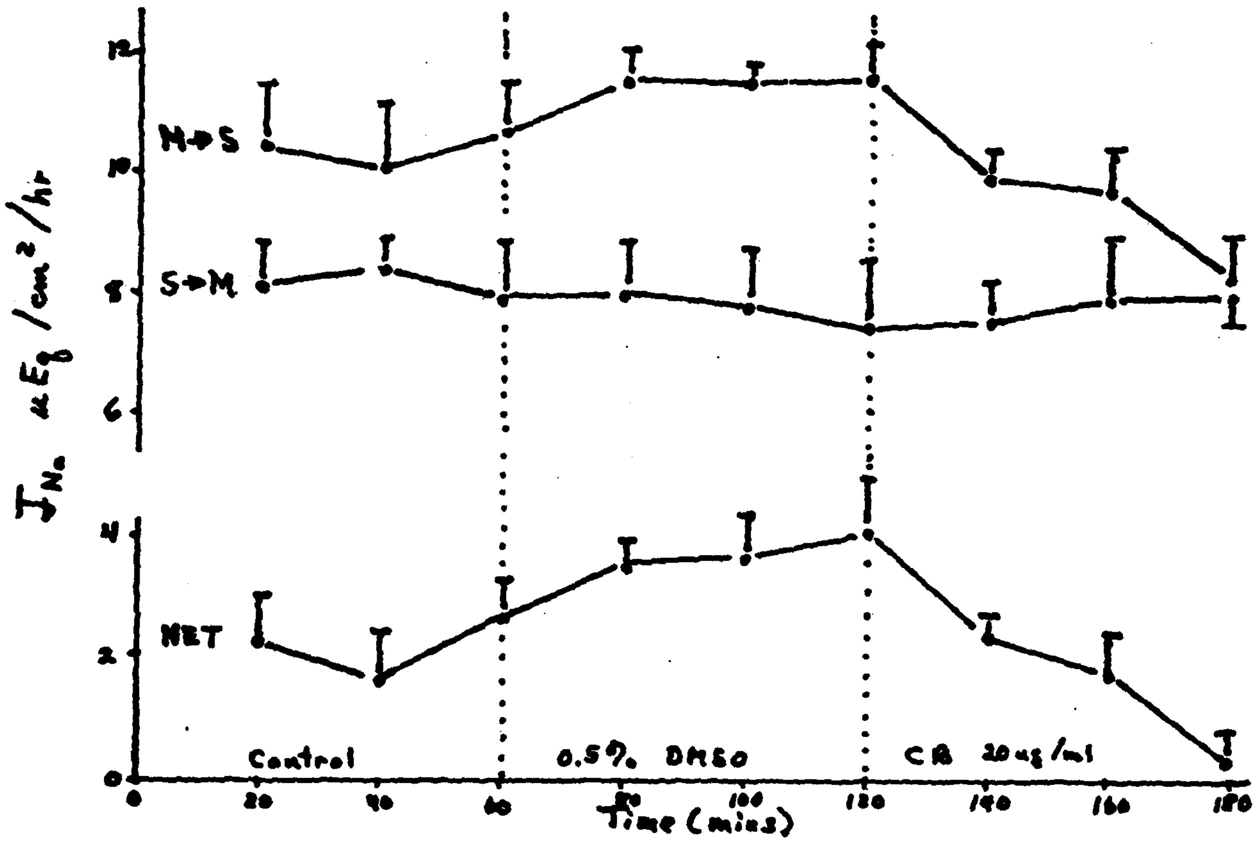
Cytochalasin B causes an inhibition of almost all active transport of sodium (and therefore water) across the rat small intestine in vitro.

Graph A



Graph A

Graph 5



J_{Na} µEq /cm² /hr
M→S
S→M
NET
Control
0.5% DMSO
CB 20 µg/ml
Time (mins)
0
20
40
60
80
100
120
140
160
180
2
4
6
8
10
12

d. Electron Microscope Results

Pieces of intestine were fixed, sectioned, and examined for all periods.

(1) Control tissues with or without DMSO showed normal intestinal morphology even after 3 hrs 40 min of perfusion.

(2) Tissues after cytochalasin B treatment did not show any evidence of gross morphological change or the breakdown of tight junctions.

(3) Using thin sections, it was not possible to determine the disruptive effect of cytochalasin B on the intracellular actin filament network.

(4) The use of potassium ferrocyanide fixative gave high contrast for actin, microtubules, and tight junctions

Conclusion

These results are consistent with the constant R value and the constant S→M passive ion movements.

e. General Conclusions

These results are consistent with the interpretation that cytochalasin B inhibits water and sodium active transport in the rat small intestine by a mechanism which involves actin filament organization.

II. Preliminary Tests of Taxol -- A Microtubule Stabilizing Agent

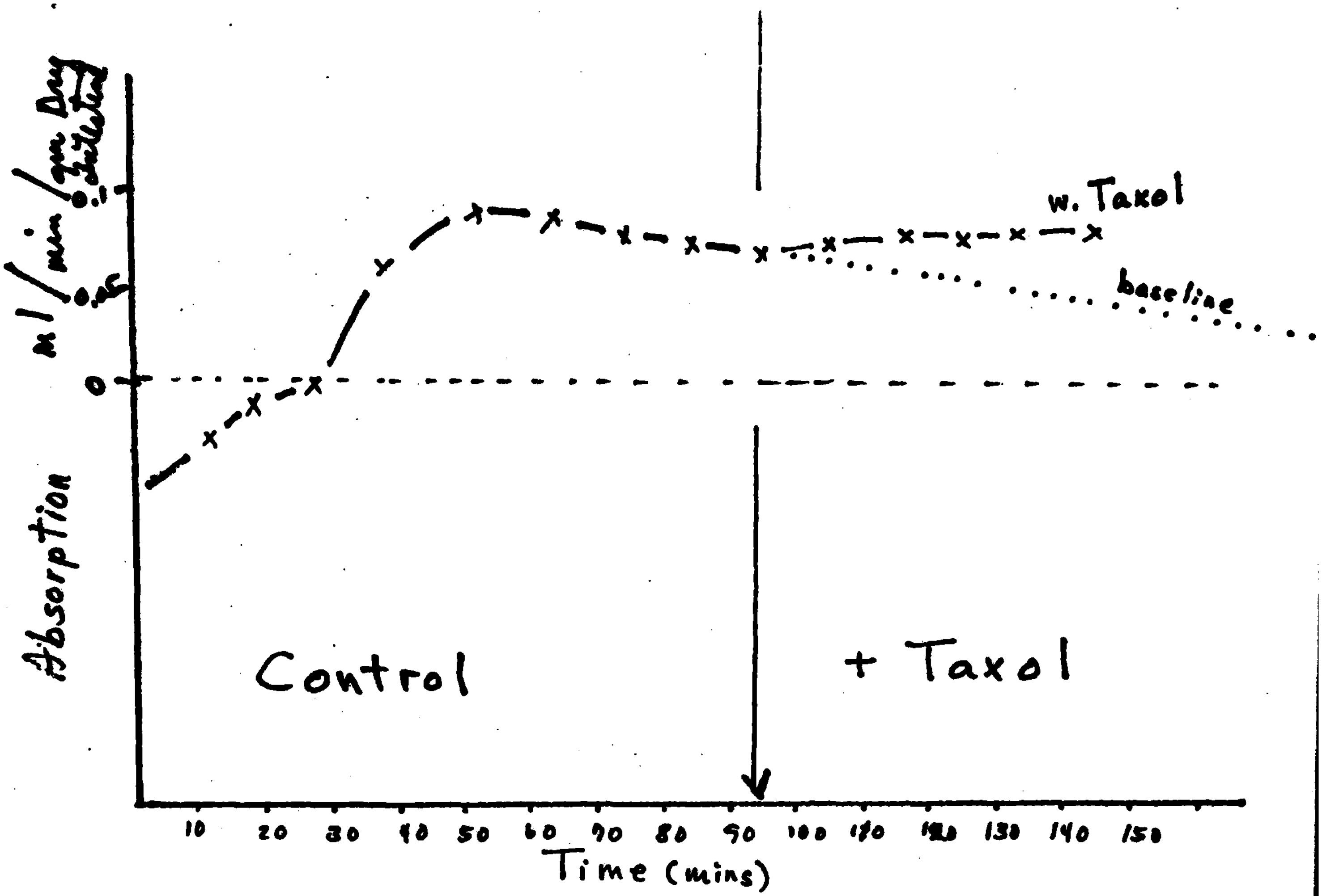
Continuing in the vein of previous logic, all drugs are being tested first by internal perfusion through the intestine of live rats. Their ability to affect transport is first screened in this way before more direct tests of the mechanism of action are begun.

Taxol, a plant product from Taxus brevifolia, was shown by Schiff et al (Nature [Lond] 277: 665-667, 1979) to promote the assembly of microtubule subunits into the polymerized microtubule form. In cultured cell systems, taxol may stabilize microtubule assemblies (PNAS 77: 1561-1565, 1980), may cause an increase in visible microtubules (J Cell Biol 91: 479-487, 1981), or may increase the probability of cross linkage between microtubules and the other cytoskeletal proteins, actin and myosin (J Cell Biol 90: 300-308, 1981). We propose that taxol may have an effect on the transport of water and salt in the small intestine by driving the structural proteins into their polymerized form.

Preliminary Results

a. In Vivo Testing

In the perfused rat small intestine, the drug taxol causes a small increase in baseline values for water absorption at 20 μg/ml dosage (see Graph C).



Rat Small Intestine in vivo plus 50ug/ml Taxol

Graph C

b. In Vitro Testing

In two experiments on rat small intestine in the Ussing chamber, taxol at 20 μg/ml and 50 μg/ml showed no effect on PD, ISC, or R over 1 hour at 37° C.

c. Minority Student Summer Program

These early experiments with taxol are being conducted by Ms. Carlett Reed, a junior year student at Grambling College. Ms. Reed is a black pre-med student who was selected on the basis of her academic excellence to participate in the Baylor College of Medicine Summer Student Program. Her stipend is fully paid by the College while she is working with Dr. Beall on this program.

d. General Conclusions

At 20 μg/ml and 50 μg/ml doses, taxol does not demonstrate an effect on water and sodium transport in rat small intestine.

III. Experiments in Isolated Enterocytes

After spending some time on the formalities, regulations, and disposal arrangements for the use of ^{22}Na isotopes in the lab at Baylor, it is now possible to attempt to measure the influx and efflux rates for sodium in the isolated intestinal enterocytes with and without the effects of cytoskeletal disrupting agents.

a. Experimental Protocol

(1) Intestinal enterocytes were isolated from sections of small intestine into L-15 tissue culture medium bubbled with O_2 at 37° C, by either mechanical agitation or collagenase digestion.

(2) Cells were pooled and incubated in a disposable plastic culture vessel containing a stir bar.

b. Results

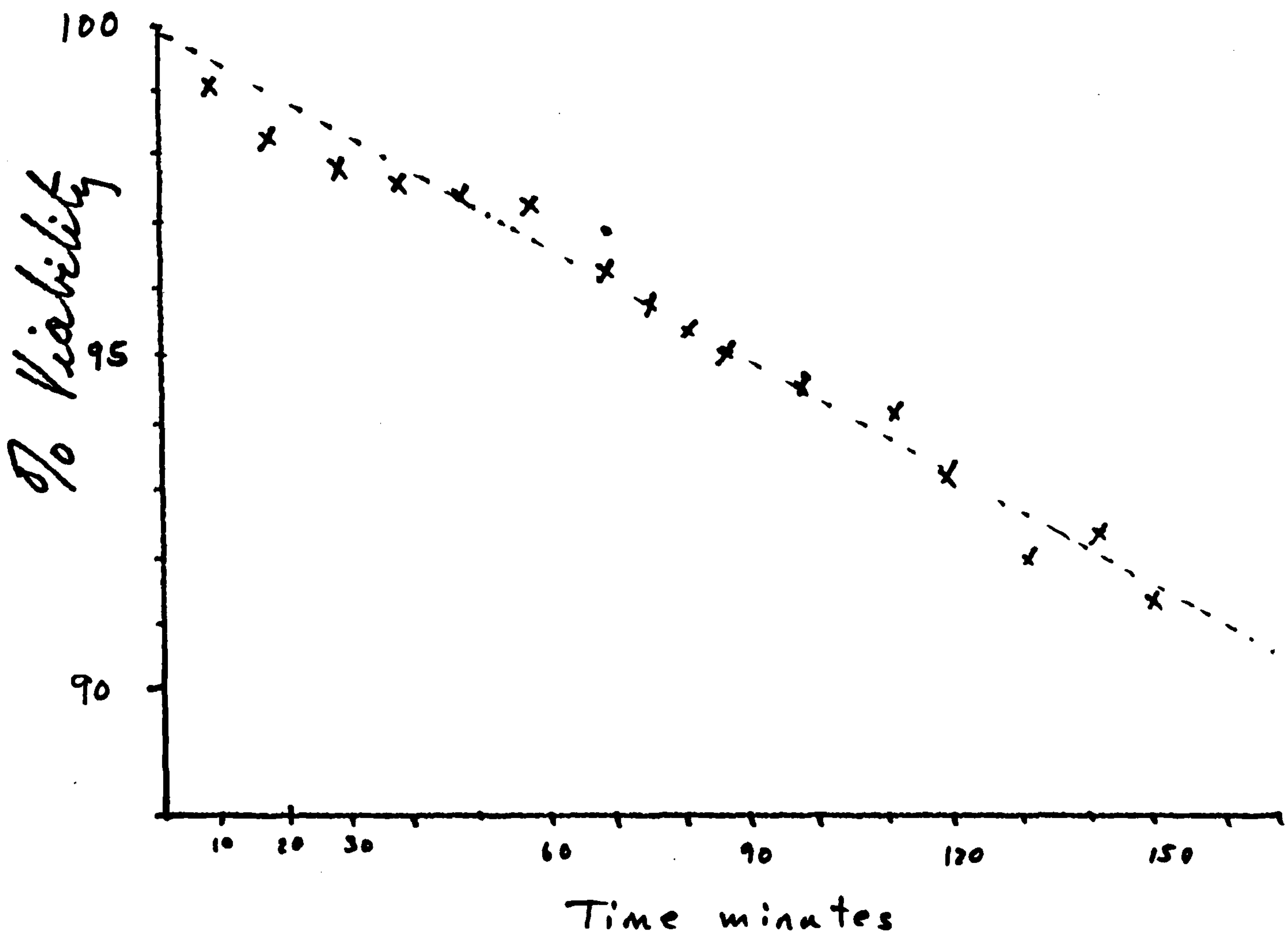
Aliquots of cells were taken at 10 min intervals and stained with trypan blue to determine % viable cells. (See Graph D.)

c. Conclusion

Cells isolated by these techniques can maintain high viability of $>$ 90% for up to 2 1/2 hrs under these conditions. They will be suitable for ^{22}Na flux studies.

IV. Plans for Year 3 -- Contract N00014-81-K-0167

In the first 20 months of this contract, the general premise that the structural proteins of the cytoplasm may play a role in transport has been successfully proven and submitted for publication. In Year 3, emphasis will be placed on understanding the role of cytoplasmic organization in the molecular



% Viable Cells after stirring in flask at 37°C for various times

Graph D

mechanism of transport. Primary emphasis will be placed on testing the corollary that:

"Organization-disorganization cycles of the cytoskeletal system of the intestinal enterocyte regulate intracellular free calcium levels, which serve as ionic regulators of transport processes."

This idea will be tested in isolated enterocyte preparations in which sodium fluxes and free intracellular calcium will be measured.

a. Research Plan

(1) Intestinal enterocytes will be isolated and maintained under conditions tested in Years 1 and 2.

(2) Steady state influx and efflux of ^{22}Na will be measured in time dependent flux studies.

(3) A lipid soluble fluorophore, sensitive to calcium , will be added to the cells, pass into them, and be converted to a water soluble fluorophore, by internal enzymes.

(4) Fluorescence will depend on "free" intracellular calcium and can be measured by a fluorescent microscope, or in a fluorometer.

(5) Upon the addition of cytochalasin B, an increase in fluoroescence will indicate a release of bound calcium on polymerized cytoskeletal elements into a "free" pool.

(6) Permeability of the cells to ^{45}Ca may also be measured with and without the drug.

b. Significance

Calcium has been postulated to be an internal messenger between the mucosal and serosal sides of the enterocyte. However, previous theories which contain no role for cytoplasmic structure in transport have not had a mechanism for rapid changes in intracellular free calcium. Since polymerized cytoskeletal structures bind calcium in the polymerization reactions and release it upon depolymerization, they may act as a storehouse of calcium. Such a view reunites the cytoplasmic structure with the role of the membrane in transport. It offers an opportunity to directly correlate dynamic structural events with active physiological processes in one type of cell.

V. Publications Crediting N00014-81-K-0167

a. Journal Papers

1. P. T. Beall: States of Water in Biology. In "Symposium on Water and Adaptation," ed. John Baust. To be published in the *Journal of Cryobiology*, 1982.

2. P. T. Beall: NMR Relaxation Times of Water Protons in Cultured Cells during Freezing and under Osmotic Stress. In *Biophysics of Water*, ed. Felix Franks. Amsterdam: Elsevier, 1982.

3. P. T. Beall, B. R. Brinkley, D. C. Chang, and C. F. Hazlewood: Microtubule Complexes correlated with Growth Rate and Water Proton Relaxation Times in Human Breast Cancer Cells. Cancer Res., accepted for publication, 1982.

4. P. T. Beall: Practical Methods for Biological NMR Sample Handling. Mag. Res. Imaging, in press, 1982.

5. P. T. Beall, C. F. Hazlewood, and L. P. Rutzky: NMR Relaxation Times of Water Protons in Human Colon Cancer Cell Lines and Clones. Cancer Biochem. Biophys. 6: 7-12, 1982.

6. P. T. Beall, S. Amtey, and B. Mela: The Systemic Effect of Cancers and Other Diseases on the Relaxation Times of Human Sera. Mag. Res. Imaging, accepted for publication, 1982.

In Preparation

7. P. T. Beall, K. Karnaky, and L. L. Shanbour: Cytochalasin B Inhibition of Sodium Active Transport in Rat Small Intestine. In preparation for submission to the American Journal of Physiology.

8. P. T. Beall, L. K. Misra, H. J. Spjut, R. L. Young, H. Evans, and A. D. LeBlanc: Clomiphene can Protect against Osteoporosis in the Mature Ovariectomized Rat. Submitted to the American Journal of Physiology.

Year 1

1. P. T. Beall: Contribution of Cytoskeleton to Cellular Water Properties in Diverse Functional States. Fed. Proc. 40: 206-213, 1981.

2. P. T. Beall, S. P. Bowen, M. M. Cassidy, and M. Dinno: Effect of Structural Perturbation by Cytochalasin B on Ion and Water Transport in "Tight" and "Leaky" Epithelia. An Electrical Study. J. Physiol. (Lond.) 308: 90-91, 1980.

b. Abstracts

1. P. T. Beall, L. K. Misra, C. F. Hazlewood, and R. L. Young: Hormone Dependent Changes in NMR Water Relaxation Times in Tissues of Aged Female Rats. Biophysical Society, 1982.

2. P. T. Beall, N. N. Izzat, and M. M. Cassidy: Effects of Cytochalasin B and Colchicine on In Vivo Intestinal Absorption in Rats. FASEB, 1982.

3. A. D. LeBlanc, H. J. Evans, P. T. Beall, L. K. Misra, H. J. Spjut, and R. L. Young: Effects of Clomiphene on Total Body Calcium in Aged Oophorectomized Rats. FASEB, 1982.

4. L. K. Misra, A. D. LeBlanc, R. L. Young, and P. T. Beall: Chronic Effects of Clomiphene on the Immune Response. FASEB, 1982.

5. P. T. Beall, D. C. Chang, S. M. Skinner, B. R. Brinkley, and C. F. Hazlewood: Temperature Effects on the Microtubule Complex and the Self-Diffusion of Water in HeLa, CHO, and BHK Cells. International Cell Cycle Conference, San Antonio, Texas, 1982.

6. P. T. Beall: Water-Macromolecular Interactions as the Origin of Relaxation Time Differences between Tissues: Implications for Whole Body Imaging. International Summer School on Advances in NMR of Biological Systems, Italy, 1982.

7. P. T. Beall: The Systemic Effect of Tumors on the NMR Relaxation Times of Sera and Tissues in Animals and Humans. International Summer School on Advances in NMR of Biological Systems, Italy, 1982.

8. P. T. Beall: States of Water in Biological Systems. Cryobiology Society, 1982. (Invited opening talk of "Symposium on Water and Adaptation.")

9. P. T. Beall, C. F. Hazlewood, and D. C. Chang: Microtubule Organization and the Self-Diffusion Coefficient of Water in Baby Hamster Kidney Cells as a Function of Temperature. American Society of Cell Biology, Baltimore, Maryland, 1982.

c. Chapters in Books

1. P. T. Beall and C. F. Hazlewood: Distinctions of the Normal, Preneoplastic, and Neoplastic States by Water Proton NMR Relaxation Times. Chapter 23 of Nuclear Magnetic Resonance (NMR) Imaging, ed. E. Partain. Philadelphia: W. B. Saunders, in press 1982.

2. P. T. Beall, S. Amtey, and K. Kasturi: Biological NMR Data Book. New York: Pergamon Press, in press 1982.

d. Reports

1. Report on the International Summer School on Advances in NMR of Biological Systems, Rende, Italy, 1982. For European Scientific Notes, Office of Naval Research, London, England, 1982.